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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

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Abstract

Myasthenia gravis (MG) is an autoimmune disease affecting the neuromuscular transmission (NMT) due to autoantibodies raised against muscle-type nicotinic acetylcholine receptors (nAChR) (Lang *et al.* 2003). Therapeutic strategies to control muscle weakness and fatigability are mainly devoted to counteract excessive immune responses, with corticosteroids as first line resource. Recently, it was demonstrated that methylprednisolone (MP) increases adenosine 5'-triphosphate (ATP) release above baseline from resting motor endplates, which anticipates adenosine (ADO) accumulation at the synaptic cleft thus contributing to amplify NMT via the activation of presynaptic facilitatory adenosine A_{2A} receptors ($A_{2A}R$) (Oliveira *et al.* 2015a). Since endogenous ADO generated during nerve stimulation in myasthenic motor endplates, is insufficient to sustain transmitter release demand through tonic activation of $A_{2A}R$ (Oliveira *et al.* 2015b), we hypothesized that benefits of corticotherapy (recovery from neuromuscular failure) in myasthenics, may result from the rehabilitation of the ADO $A_{2A}R$ tonus, leading to increases the acetylcholine (ACh) release and muscular strengthening.

In this study, we used *Wistar* rats with experimental autoimmune *myasthenia gravis* (EAMG), which were immunized with the R97-116 peptide, a synthetic peptide corresponding to a specific region on the α subunit of the rat nicotinic AChR, made up in a solution containing the Complete Freund's Adjuvant (CFA) (Oliveira *et al.* 2015b). Thirty days after the first inoculation, the animals were boosted with the R97-116 peptide made up with the Inactive Freund's Adjuvant (IFA). Control animals received the CFA emulsion without the peptide. Animals from the Naïve group were not submitted to treatment. Clinical scoring was based on the presence of tremor, hunched posture and fatigue. Muscle strength was assessed by the grip strength test (BIOSEB, France).

Pre-treatment of hemidiaphragm preparations isolated from EAMG rats with methylprednisolone 300 µM (MP 300 µM) significantly increased acetylcholine ([^{3}H]-ACh) release evoked by phrenic nerve stimulation with 50 Hz Bursts. No significant changes (p>0.05) were detected, when comparing the facilitatory effect of MP (300 µM) in EAMG (33±14%, n=5) with Control (24±3%, n=6) and Naïve (40±11% n=5). Despite the reported loss of $A_{2A}R$ tonus in EAMG rats (Oliveira *et al.* 2015b) amplification of NMT induced by MP (300 µM) is dependent of $A_{2A}R$ activation, because the facilitatory effect of MP (300 µM) was prevented by the $A_{2A}R$ antagonist, 4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol (ZM 241385) (50 nM). Like in both Naïve and Control animals, MP (300 µM) also significantly (p<0.05) increased the outflow of ATP in resting motor endplates of EAMG rats from a basal level of 526±71 pM to 794±71 pM (n=5) using the luciferin-luciferase bioluminescence assay.

Data show here, for the first time, that MP can rehabilitate NMT failure in EAMG animals, by increasing the outflow of ATP from resting motor endplates, which upon its extracellular hydrolysis into ADO may enhance the $A_{2A}R$ tonus leading to facilitation of evoked transmitter release during high-frequency nerve firing.

Abbreviations

α-BTX - α-bungarotoxin

A_1R - Adenosine A_1 receptor

$A_{2A}R$ - Adenosine A_{2A} receptor

A_3R - Adenosine A_3 receptor

Abs - Antibodies

ACh - Acetylcholine

Acetyl CoA - Acetyl coenzyme A

AChE - Acetylcholinesterase

AChR - Acetylcholine receptor

ADA - Adenosine deaminase

ADO - Adenosine

ADP- Adenosine 5'-diphosphate

AMP - Adenosine 5'-monophosphate

ATP - Adenosine 5'-triphosphate

BID - Twice a day

cAMP - Cyclic denosine 5'-monophosphate

CD73 - Ecto-5´-nucleotidase

ChAT – Choline Acetyltransferase

CFA - Complete Freund's adjuvant

CMAPs - Compound muscle action potentials

DPM - Disintegrations per minute

EAMG - Experimental Autoimmune Myasthenia Gravis

h - Hour

[^{3}H]-ACh - Evoked Acetylcholine

IFA - Incomplete Freund's adjuvant

Ig - Immunoglobulins

IMP - Inosine monophosphate

INO - Inosine

ip - Intraperitoneal

im - Intramuscular

iv - Intravenous

Kg - Kilogram

LEMS - Lambert-Eaton myasthenic syndrome

LRP4 - Low density lipoprotein receptor-related protein 4

L-type - presynaptic Ca_V1 voltage-gated calcium channels

M_1R - Muscarinic M_1 receptor

M_2R - Muscarinic M_2 receptor

MHC II - Major histocompatibility complex class II

MIMV - Mestrado Integrado em Medicina Veterinária

mM - MiliMolar

mm - Millimeters

min - Minute

mL - Milliliter

mg/Kg - Milligram per kilogram

n - Sampling

nAChR - Nicotinic acetylcholine receptor

NMJ - Neuromuscular junction

nmol/L - Nanomol per liter

NMT - Neuromuscular transmission

mA - Milliamps

MG - *Myasthenia Gravis*

MP - Methylprednisolone

ms - Milliseconds

MuSK - Muscle-specific kinase

PBS - Phosphate Buffered Saline

pi - Post-immunization

Pm - PicoMolar

po - *per os*

P/Q-type - Presynaptic $Ca_v2.1$ voltage-gated calcium channels

QID - Four times a day

QOD - Once every 48 hours

R97-116 - Syntetic peptide corresponding to region 97-116 of the rat nAChR α subunit

RIA - Immunoprecipitation radioimmunoassay

SNARE - N-ethylmaleimide sensitive factor attachment receptor complex

SEM - Mean standard error

sc - Subcutaneous

SPF - Sociedade Portuguesa de Farmacologia

SVs - Synaptic vesicles

TID - Three times a day

TIMG - Toxin-induced Myasthenia Gravis

UP - Universidade do Porto

VGCC - Voltage-gated calcium channels

VGSC - Voltage-gated sodium channels

VGKC - Voltage-gated potassium channels

ZM241385 - 4- (2- (7-amino-2- (furan-2-yl) - [1,2,4] triazolo [1,5-a] [1,3,5] triazin-5-ylamino) ethyl)phenol

µCi - MicroCurie

µg - Microgram

µL - Microliter

µM - MicroMolar

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Part of this work was presented as an oral communication at the Annual Meeting of the "Sociedade Portuguesa de Farmacologia (SPF)" (Lisbon 2015)

- Oliveira L, Mota C, Fernandes M, Neves L, Silva I, Correia-de-Sá P (2015) "Methylprednisolone rehabilitates the presynaptic facilitatory adenosine A_{2A} receptor tonus in the neuromuscular junction of rats with experimental autoimmune Myasthenia gravis" **SPF** 5-6 February 2015 Lisbon

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2. Introduction

2.1. *Myasthenia Gravis* – "Myasthenia" Greek (muscle weakness) and "gravis" Latin (severe)

Acquired *Myasthenia Gravis* (MG) is a relatively rare autoimmune neuromuscular disorder, clinically characterized by muscle weakness and fatigability, as a result of the production of autoantibodies against proteins of the postsynaptic membrane in the neuromuscular junction (NMJ). About 85% of patients with MG have immunoglobulin G (IgG) autoantibodies against binding sites on the nicotinic acetylcholine receptor (nAChR) (Lindstrom *et al.* 1976; Shigemoto *et al.* 2006; Cavalcante *et al.* 2011; Aviden *et al.* 2014). Other patients have autoantibodies against muscle-specific kinase (MuSK) (8%) and low-density lipoprotein receptor-related protein 4 (LRP4) (5%) (Berrih-Aknin *et al.* 2014; Huijbers *et al.* 2014). It is also described a distinct myasthenic syndrome, Lambert-Eaton myasthenic syndrome (LEMS) (2%), an autoimmune disorder characterized by antibodies (Abs) raised against the presynaptic $Ca_v2.1$ voltage-gated calcium channels (VGCC) (Huijbers *et al.* 2014; Ha & Richman 2014).

There are multiple mechanisms that explain how nAChR autoantibodies interfere with the normal function of the NMJ: (1) complement-mediated inflammatory destruction of the post-synaptic membrane of skeletal muscle cells (Tüzün *et al.* 2003); (2) antigenic modulation by cross-linking of the target antigen; competition at ligand-binding sites; (3) steric hindrance that inhibits conformational changes interactions with associated molecules, reducing the nAChR number on muscle fibers and consequently decreasing neuromuscular transmission (NMT) (Ha & Richman 2014). The relative contribution of these mechanisms to the pathophysiology of all forms of MG depends on the epitope specificity of the autoantibody and the immunoglobulin subclass involved (Huijbers *et al.* 2014). The T lymphocytes lead the attack to motor endplates, by recognition of the antigen coupled to the major histocompatibility complex class II (MHC II), thus promoting B lymphocytes production of anti-AChR (primarily isotypes IgG1 e IgG3) Abs. (Rodgaard *et al*. 1987; Aviden *et al*. 2014; Huijbers *et al*. 2014; Ha & Richman 2014).

Epidemiological analysis of human patients with MG exhibiting autoantibodies against AChR revealed a bimodal distribution: early onset MG – with female predominance at 20-40 years of age (Carr *et al.* 2010); and late onset MG – with male predominance at 60-80 years of age, and in this group there is a higher rate of association with thymoma (Muller-Hermelink & Marx 2000; Huijbers *et al*. 2014). Between the ages of 50-60 there is no gender difference in predominance of MG (Carr *et al.* 2010; Berrih-Aknin *et al.* 2014). The disease usually begins with ocular symptoms and extends to other muscles in 80% of cases. It also includes other features: variability, effort induced worsening, successive periods of exacerbation during the course of

the disease, severity dependent on respiratory and swallowing impairment (myasthenia crisis if acute evaluation). The diagnosis is based on the clinical features, the benefit of the cholinesterase inhibitors (hydrophonium or edrophonium intravenous (iv) has a brief response in 3-5 minutes (min); neostigmine subcutaneous (sc)/intramuscular (im) from 15 min to 2 hours (h) reverts ptosis, hypernasal voice, limb weakness), the detection of autoantibodies and significant decrement evidenced by electrophysiological tests (Berrih-Aknin *et al.* 2014).

Reestablishment of NMT is the main goal for the current MG therapeutics and could be achieved by two different approaches. Symptomatic therapy is often needed, which includes cholinesterase inhibitors that partially compensate for the reduced safety margin of NMT acting postsynaptically. However, the increasing ACh bio-availability induced by acetylcholinesterase (AChE) inhibitors has some undesirable systemic side effects, like autonomic and (cholinergic crisis very similar to MG crisis). Another strategy of treatment is based on early intervention with immune suppression to limit epitope spread and long-term disease severity, like plasmapheresis, thymectomy, iv administration of immunoglobulins and immunosuppressive drugs, such as corticosteroids and azathioprine, to inhibit humoral and cellular immune responses in order to reduce skeletal muscle destruction (Berrih-Aknin *et al.* 2014; Huijbers *et al.* 2014).

Safer and specific therapies in myasthenic syndromes are still required, because of the potential for serious side effects associated with present broad immunosuppressive and anticholinesterase therapies.

2.2. *Myasthenia Gravis* therapeutic approach in Veterinary Medicine

Many of the newest therapeutic options available in veterinary medicine for MG, are based on current strategies used in Humans with this disease (Khorzad *et al.* 2011). MG is likely the most commonly diagnosed neuromuscular disease in small animal practice, and is relatively common in dogs. While diagnosing and treating dogs and cats with MG is rewarding, it can be devastating if not recognized early and treated appropriately. Ormrod described MG in a dog in 1961 and Dawson in a cat in 1970. The autoimmune form of canine MG was described in 1978 by Lennon and collaborators (reviewed in Vernau 2009). Acquired MG is the most commonly recognized immune-mediated neurological disease affecting dogs and occasionally cats (Shelton 2002; Platt & Olby 2004). “Acquired MG has been observed in dogs older than 3 months, of all breeds but particularly in German Shepherd Dogs, Golden Retrievers and Labrador Retrievers. In one report, the relative risk of acquired MG in different breeds of dogs was highest in Akitas (Shelton *et al.* 1997; Andrade *et al.* 2007). Newfoundlands may also be predisposed to acquired MG” (Platt & Olby 2004), as well as Great Danes (Dewey *et al.* 1988-

1995; Lorenz *et al.* 2011), German Shorthaired Pointer and Chihuahua (Shell 2012). Machado & Brizzotti (2012) reported a case of acquired MG in a poodle. A bimodal age of onset, like in Humans has also been reported in affected dogs (<5 - >7 years) (Platt & Olby 2004; Shell 2012). In one review of cats with acquired MG, gender was not a risk factor (Shelton *et al.* 2000; Platt & Olby 2004). There is a higher incidence in Abyssinians, Somalis and domestic shorthair cats (Lorenz *et al.* 2011; Machado & Brizzotti 2012).

Acquired MG is classified based on distribution and severity of clinical signs, and can be classified into 4 major groups, those with only focal clinical signs without generalized weakness, mild cases with a progressive generalized weakness in the absence of megaesophagus, those with a more severe form - fulminant - acute generalized weakness and megaesophagus, or as a paraneoplastic syndrome associated with thymoma (Abelson *et al.* 2009; Khorzad *et al.* 2011).

In focal MG, there is no clinical evidence of thoracic or pelvic limb muscle weakness; instead weakness occurs in 1 or more muscle groups including the facial, esophageal, pharyngeal and laryngeal muscles. A dog may present only megaesophagus, being the regurgitation or dysphagia the only clinical signals (36 – 43 % of all canine cases) (Khorzad *et al.* 2011), while in cats megaesophagus and dysphagia occurs in approximately 15% of cases (Platt & Olby 2004). This is explained by the composition of the muscularis externa in the esophagus. In the dog the muscular externa is mainly composed by skeletal muscle, while in the cat, the muscularis externa is composed by skeletal muscle throughout much of the esophagus, but in the caudal one-fifth there is a transition to smooth muscle (Bacha & Bacha 2000). In Humans, where only the proximal 2 – 6 centimeters of the muscularis externa of the esophagus is skeletal muscle, with the remainder being smooth muscle, megaesophagus is not common (Abelson *et al.* 2009). Fifteen percent of people have focal MG restricted to the eyes (Khorzad *et al.* 2011). Generalized MG can manifest in a wide range of clinical signals, ranging from mild to severe weakness to megaesophagus. In animals, the pelvic limbs are more affected than the thoracic limbs. Generalized MG occurs in 57 – 64 % of all canine cases, with 90% of them having megaesophagus (Khorzad *et al.* 2011), while generalized weakness without megaesophagus occurs in approximately 30% of cats and generalized weakness and megaesophagus/ dysphagia occurs in 20% of cats (clinical signals in this species is more variable) (Platt & Olby 2004; Lorenz *et al.* 2011). An acute fulminant form of MG with a rapid onset of paralysis and megaesophagus, frequent episodes of regurgitation with secondary aspiratory pneumonia, and respiratory distress, occurs in less than 5% of dogs, compared to the 12% of cases in Humans, identified in a study conducted in China by Yu and collaborators (Yu *et al.* 1992; Khorzad *et al.* 2011). MG may be developed as a part of a paraneoplastic syndrome, and affected animals may display clinical signs related to a primary neoplasm (thymomas, osteogenic sarcoma,

cholangiocellular carcinoma, anal sac adenocarcinoma and lymphoma). MG has been identified in 30 – 50 % of dogs with thymomas but the incidence of thymoma associated with MG is higher in cats than in dogs (Lorenz *et al.* 2011; Khorzad *et al.* 2011). It is referred that the incidence of mediastinal thymoma occurs in 25.7% of cats with MG, and in dogs the incidence is 3.4% (Platt & Olby 2004). In Humans 30 – 60% of thymomas are associated with MG according to Vincent and collaborators (Vincent *et al.* 2001; Khorzad *et al.* 2011).

The diagnosis of MG begins with the correct evaluation of the patient, proper anamnesis, physical examination, including neurological examination, which should lead to suspect from MG. Clinical signs usually consist of all or some of the following: exercise-induced weakness (after rest they walk normally for a short period before the stride shortens, until they crouch in sternal recumbency and rest their head on their forepaws), fatigue, pelvic limb weakness or stiffness, megaesophagus, reduced gag response, voice change, neck weakness, and facial weakness (Shell 2012). Many cats have facial weakness, with dropped jaw, are unable to close their eyes and manifest ventro-flexion of the neck (Platt & Olby 2004; Lorenz *et al.* 2011). A presumptive diagnosis of MG may be made by the resolution of muscle weakness following an iv injection of edrophonium chloride. Thorax radiography is indicated to detected secondary aspiration pneumonia, cranial mediastinal mass, megaesophagus and pneumonia or pneumonitis (Shell 2012). Currently, serum Abs against nAChR (MG titer) is the “gold standard” test for diagnosing acquired MG by immunoprecipitaion radioimmunoassay (RIA); this test is specific, sensitive and an nAChR Abs titer >0.6 nanomol per liter (nmol/L) is diagnostic in dogs (Khorzad *et al.* 2011) and >0.3 nmol/L is positive in cats (Shelton 2010). To date, Abs against muscle-specific kinase (MuSK) have been identified in only one MG canine, which was seronegative to nAChR Abs (Shelton 2010). Similar to Humans, Shelton and collaborators in 2001 described cases of canine MG with autoantibodies against skeletal muscle striations in dogs with thymomas, against titin in older-onset MG and against ryanodine receptor in severe forms of thymoma-associated MG (Shelton 2010). Repetitive nerve stimulation, performed under general anesthesia (which can present serious risks for the patient), can be used to support the diagnosis of MG, especially in cases that have negative nAChR Abs test results. Findings that support the diagnosis of MG are: reduced amplitude of compound muscle action potentials (CMAPs), decrease of the CMAPs with repetitive stimulation (and its reversal with an iv administration of edrophonium) (Shell 2012).

Shelton and Lindstrom, in 2001, confirmed that in the absence of immunosuppression, the natural course of canine MG is for clinical and immune remission (clinical signs resolved and Abs titters within the reference interval) and therefore immunosuppressive drugs should not be

used unless in severe cases of MG, which do not respond to AChE treatment alone (Shelton 2010).

In acquired MG, there are three major modalities of therapeutic intervention: anticholinesterase therapy, immunomodulatory therapy, and thymectomy. In Veterinary Medicine, anticholinesterase therapy is most widely accepted. Long-acting anticholinesterase drugs prolong the action of ACh at the NMJ by reversibly inhibiting AChE. The agent most often used is pyridostigmine bromide (Mestinon®, 0.5 to 3 milligram per kilogram (mg/Kg) every 8 h (QID) to 12 h (BID) *per os* (po)). To avoid overstimulation of AChR, treatment is started at the low end of the dosage range, and the dose is gradually increased to effective dosages (Lorenz *et al.* 2011). Patients that cannot tolerate the oral form, because of frequent regurgitation from megaesophagus are treated with neostigmine bromide, which is administered at 0.04 mg/kg four times a day (QID) im (Abelson *et al.* 2009). The dosages are titrated to an optimal level based on changes in muscle strength. Some dogs with megaesophagus may simply require elevated feedings, while some may require placement of a gastrotomy tube (in case of intractable regurgitation, even with altered feeding techniques). Generally dogs and cats should be feed two to three times daily, in an elevated (standing or upright sitting) position for 20-30 min, after each feeding. Joe and Donna Koch developed the "Bailey Chair", where the dogs are comfortable, can eat, drink, receive medications, and remain in a stable, upright position following a meal. The consistency of the food (liquid, solid, gruel) may also need to be altered depending on which is handled best (Shell 2012). Cats are sensitive to anticholinesterase drugs, and do not appear to suffer from the muscular weakness secondary to corticosteroids that dogs do, particularly when high dosages are used. Therefore, the first line treatment for cats with MG is immunosuppression with corticosteroids. In dogs and cats with thymoma, complete excision results in clinical and immunological remission of MG (Vernau 2009). While there are not any controlled trials to address the optimum treatment for cats and dogs with thymomas or those that respond poorly to medications, surgical excision is currently believed to be the ideal treatment (Lorenz *et al.* 2011). It should be taken alternative treatments in case of secondary aspiratory pneumonia, severe generalized MG and fulminant MG (ventilatory support may be needed). The key to a successful outcome may be the prevention and/or aggressive treatment of aspiration pneumonia, since the mortality rate in acquired MG approaches approximately 50%, due in large part to misdiagnosis of vomiting instead of regurgitation from esophageal weakness and dilatation. In the absence of severe aspiration pneumonia, pharyngeal weakness, or acute fulminating MG, the prognosis for survival and complete remission is usually good (Shell 2012).

There are documented two case reports of acquired MG in other species, in a 14 years old female polar bear (*Ursus maritimus*) which acutely developed hind limb weakness (Kenny *et al.* 2004) and in a 7 months-old male ferret (*Mustela putorius furo*), evaluated for episodic pelvic limb weakness of 2 weeks´ duration (Couturier *et al.* 2009). These cases have clinical relevance because in the future, veterinarians with a case of a muscle weakness in these species should consider MG as a differential diagnosis, and even though the gold standard diagnosis method is relatively species-specific, it may occur some cross-reactivity in Abs recognition of the nAChRs between species, as referred in these reports.

2.2.1. Animal models to study *Myasthenia gravis*

Laboratory animal science is defined as the study of the scientific, ethical and legal use of animals in biomedical research. It comprehends a multidisciplinary field encompassing biological and pathobiological specialties for the optimal use of animals as models, either for humans, or other species. Cause, nature and cure of diseases are the main purposes for the use of these models, and in the study of MG are developed and used induced experimental disease models. It is important to have in mind the similarities and the differences of the species chosen, and the resemblance pathology and outcome of an induced disease or disorder in the model species, with the respective lesions of the target species, so the experimental results can be extrapolated from one species to the other (Hau & Van Hoosier Jr 2003). If there is an experimental model already accepted and validated for the disease to be tested (the etiology, course and pathology similarities/differences between the induced model, and humans for example, are known) it should be preferred. Few induced models completely mimic the target disease in Human´s (Hau & Van Hoosier Jr 2003).

Patrick and Lindstrom in 1973 immunized rabbits in order to obtain autoantibodies against the recently purified nAChR, and observed that the animals developed weakness and electrophysiological abnormalities that were similar to those in human MG (Patrick & Lindstrom 1973). This experimental disorder in rabbits, received lately the name Experimental Autoimmune *Myasthenia Gravis* (EAMG). Later EAMG was reproduced in other species (Lennon *et al.* 1975) and has contributed with a great deal of information for unveiling the molecular and immunological features of this disease. There are numerous procedures to create an animal model for MG. A very common one, which recreates most of the observed symptoms of MG in Humans (Attachment 1), consists on injecting rodents with anti-nAChR Abs and/or their immunization with nAChRs isolated from *Torpedo californica* (Aricha *et al.* 2006). Baggie and collaborators showed that the breaking of tolerance to a single T cell epitope of the self-autoantigen induces autoreactive T cells and specific Abs to rat AChR (Baggi *et al.* 2004). This model was established by immunizing a susceptible rat strains (*Lewis* rats) with a synthetic

peptide corresponding to region α97-116 of the rat AChR α subunit, in CFA (Complete Freund's Adjuvant – a mixture of oils and water plus killed *Mycobacterium tuberculosis* strain, used to stimulate immune response). This model of EAMG is valid as a model to understand the key immunological processes and molecular aspects, leading to MG as well as providing a practical instrument for testing the capability of possible treatment methods for MG and other antibody-mediated autoimmune diseases (Baggi *et al.* 2012).

It is also possible to induce nonimmunogenic MG in animals by administration of AChR-blocking toxins (e.g. α-Bungarotoxin) (Molenaar *et al.* 1991). In this model, *Wistar* rats are subcutaneously injected with α-bungarotoxin (α-BTX) every 48 h (QOD) for 3 weeks. α-BTX is well established as an irreversible antagonist of muscle nAChR containing α1 subunits. Several groups have presented evidences that the number of functional nAChRs in rat hemidiaphragms was significantly reduced after 2 - 3 weeks of α-BTX treatment, without evidencing a structural damage of muscle membranes (Plomp *et al.* 1992) and/or changes in the endplate AChE activity (Van Kempen *et al.* 1999). The Toxin-induced Myasthenia Gravis model (TIMG) (Molenaar *et al.* 1991) is used to better understand the underlying molecular mechanisms behind the NMT deficit.

2.3. The neuromuscular junction

The NMJ is a special type of synapse designed to efficiently transmit electrical impulses from myelinated motor nerves to the skeletal muscle cells. This synapse has three major structural elements: the motor neuron (or presynaptic region), the skeletal muscle fiber and the perisynaptic Schwann cell. The primary specializations include a terminal Schwann cell that caps the motor nerve, terminal branches of the motor axon, which accumulate mitochondria´s (provide energy for the synthesis and release of ACh, and microtubules) and synaptic vesicles (SVs), and an AChR-rich postsynaptic endplate (Hughes *et al.* 2006), where vesicles fuse with the membrane and release their contents into the synaptic cleft. In the presynaptic region there are also voltage-gated calcium channels (VGCC) of the P/Q- (presynaptic $Ca_V2.1$ voltage-gated calcium channels) and L-type (presynaptic Ca_V1 voltage-gated calcium channels) subtypes (Correia-de-Sá *et al.* 2000).

The NMJ begins to form when the axon growth cone of developing motor neuron, or a sprouting motor axon, encounters a developing myotube, or a denervated muscle fiber, and begins to secrete a glycoprotein – agrin – with a laminin-binding domain that anchors it to the extracellular matrix, but requires the presence of postsynaptic transmembrane kinase, the muscle-specific kinase (MuSK). The agrin/MuSK interaction requires mutual binding to a third transmembrane muscle protein, the low density lipoprotein receptor-related protein 4 (LRP4). This process

induces dense clustering of the AChRs in the postsynaptic membrane and marked folding and specialization of that membrane (Hughes *et al.* 2006; Ha & Richman 2014). In the mature NMJ appear secondary specializations involving the formation of active zones along the junctional surface, distribution of the organelles asymmetrically, inside the nerve terminal, to the synaptic cleft, and the formation of secondary synaptic clefts, that creates folds in the postsynaptic membrane, where the nAChRs (ligand-gated ion channels) are clustered, while voltage-gated sodium channels (VGSC) concentrate in the depths of theses folds and also throughout the muscle membrane (Hughes *et al.* 2006). Stabilizers of the NMJ, such as proteins and proteoglycans, and the enzyme acetylcholinesterase (AChE) are also present in the synaptic cleft (Martyn *et al.* 2009) (Figure 1).

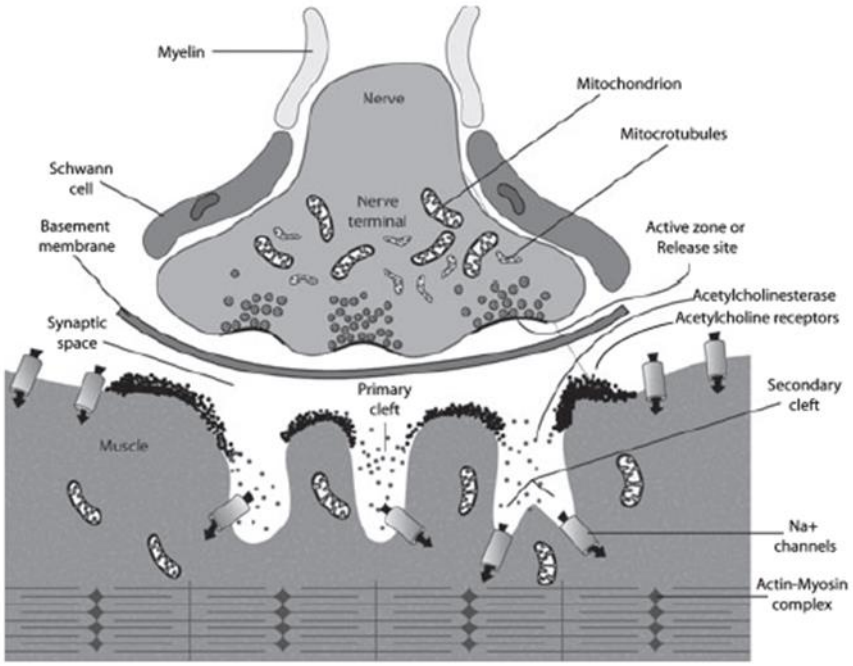

Figure 1 - The mature NMJ and its main components: the nerve terminal (presynaptic region), the perisynaptic Schwann cell and the muscle fiber (specialized postsynaptic membrane). All 3 parts contain organelles and molecules not found in/or preferentially expressed when compared with extrasynaptic regions. (Martyn *et al.* 2009).

Muscle nAChRs cooperate to facilitate fast NMT. Nicotinic receptors are pentameric transmembrane proteins, made up of combinations of 5 individual subunits (α, β, γ, δ and ε) that surround a central pore. In the adult NMJ, the stoichiometry of the muscle-type nAChRs is α2βδε. The binding of two molecules of ACh at the N-terminal domain of the interface between α/ε and α/β subunits induces a conformational change allowing the influx of sodium, thus leading to depolarization of the skeletal muscle membrane. Propagation of the action potential requires subsequent opening of voltage-sensitive sodium channel-rich valleys of the synaptic folds (Hughes *et al.* 2006; Ha & Richman 2014) (Figure 2-A). The rat NMJ is equipped with

α3β2 neuronal nAChR, which mediate the facilitation of ACh release, in addition to muscle-type nAChR containing the α1 subunit (Faria *et al.* 2003).

After the initiation of an action potential, voltage-sensitive calcium channels open and the rapid increase in free Ca^{2+} in the nerve terminal initiates the mobilization and docking of SVs near active zones (fusion with the presynaptic membrane), which involve phosphorylation of synapsin and the formation of the soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) complex and, subsequent, release of ACh into the synaptic clefts (Hughes *et al.* 2006) (Figure 2-B).

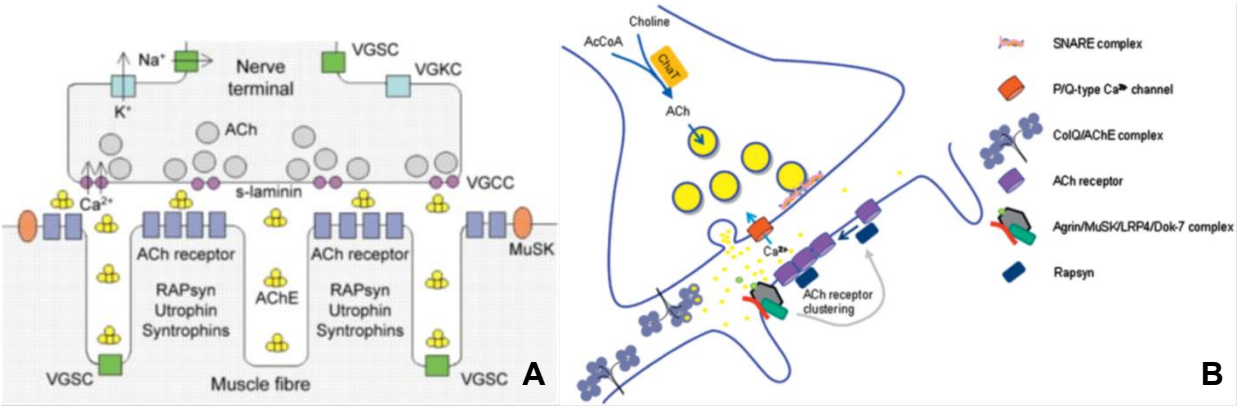

Figure 2 – The mature NMJ: **A** – VGSC= voltage-gated sodium channel, VGKC= voltage-gated potassium channel, VGCC= voltage-gated calcium channel; **B** – Schematic of MuSK and associated molecules responsible for nAChR clustering. (Ha & Richman 2014)

The released ACh activates the nicotinc receptors (nAChRs) and the transmitter is rapidly hydrolyzed to acetic acid and choline by AChE in order to prevent continuous stimulation of nAChRs, preventing receptor desensitization, and allowing a higher safety margin of NMT. The choline is transported back into the nerve terminal, via sodium-dependent high-affinity choline transporters, and is re-used to synthesize new molecules of ACh by its combination with acetyl coenzyme A (Acetyl CoA), a reaction that is catalyzed by choline acetyltransferase (ChAT), which is produced in the cholinergic cell body and transported down the axon to the nerve endings (Hughes *et al.* 2006).

Diseases of NMJ produce weakness which generally varies with repeated synaptic firing, sustained muscle contraction or repeated muscle contraction. The “true” myasthenias are characterized by weakness, which worsens with sustained muscle contraction or work and improves with rest. It has been determined that they primarily involve components of the postsynaptic portion of the NMJ and can be confirmed electrophysiologically by decrementing CMAPs, in response to slow rates of motor nerve stimulation (2-3 Hertz (Hz)), in association with normal amplitudes of the responses in case of a single nerve stimuli (Ha & Richman 2014).

2.4. Adenosine receptors in healthy and myasthenic motor endplates

Adenosine (ADO) is a nucleoside involved in processes of the primary metabolism, such as the modulation of cellular metabolic state, and plays a modulatory role in the NMT, as it can be released from activated nerve terminals, from Schwann cells and from activated muscle fibers (Cunha 2005). ADO at the NMJ can be obtained by the catabolism of adenine nucleotides, when it is co-released with ACh from motor nerve terminals (Correia-de-Sá & Ribeiro 1996). The rate limiting step for ADO production from the hydrolysis of adenosine 5'-triphosphate (ATP) is dephosphorylation of adenosine 5'-monophosphate (AMP) by the ecto-5'-nucleotidase (CD73) (Cunha *et al*. 1996). Another source of extracellular ADO is cyclic adenosine 5'-monophosphate (cAMP), which can be released from various cells including neurons, converted into AMP by extracellular phosphodiesterases, and thereafter into ADO by ecto-5´-nucleotidase (CD73) (Fredholm *et al.* 2001). Magalhães-Cardoso and collaborators (2003) showed that there is a shunt in the ADO formation at the rat NMJ, with the conversion of AMP into inosine monophosphate (IMP) by the enzyme ecto-AMP deaminase. Then, IMP is hydrolyzed into inosine (INO) by CD73, without ADO formation or the intervention of adenosine deaminase (ADA).

Extracellular ADO initiates transmembrane signaling via four G protein-coupled receptors subtypes, bound to the plasma membrane, A_1, A_{2A}, A_{2B} and A_3. These receptors are structurally distinct and are encoded by four different genes. They are differentiated based on their coupling to cAMP production via the adenylate cyclase system. Cyclic AMP levels are dependent on the type of G protein coupled to the receptor: A_1 and A_3 receptors are coupled to Gi protein inhibiting adenylate cyclase, which leads to an intracellular decrease of cAMP levels. A_{2A} and A_{2B} receptors, are coupled to Gs protein, stimulate adenylate cyclase, which leads to an increase of cellular cAMP levels (Fredholm *et al.* 2001). ADO action depends on the receptor density, affinity and location, and in general the agonist is more efficient, when higher densities of the receptors are present. Therefore, low endogenous ADO levels, the ones observed under basal conditions, have the potential to activate the receptors only when they are in higher numbers, and not when these receptors are sparse (Fredholm *et al.* 2001). At the rat NMJ, high-affinity A_1 and A_{2A} receptors are responsible for the major effects exerted by the ADO, namely at modulating synaptic transmission. Co-existence of both inhibitory A_1R and facilitatory $A_{2A}R$ on the same nerve terminal was first proved using neurochemical and electrophysiological methods at the rat NMJ (Correia-de-Sá *et al.* 1991); later on it was shown that ADO could facilitate the release of neurotransmitter via activation of cAMP-coupled $A_{2A}R$ (Correia-de-Sá & Ribeiro 1994). The dual modulatory role of ADO via presynaptic inhibitory A_1R and facilitatory $A_{2A}R$ is highly dependent on the nerve stimulation pattern (Correia-De-Sá *et al.* 1996),

particularly when this nucleoside is build-up from the catabolism of ATP release (Magalhães-Cardoso *et al.* 2003). The tonic inhibitory effect mediated by A_1R, is observed at low frequency stimulation under resting conditions, where low amounts of ADO activate predominantly inhibitory A_1R. High-frequency, high-intensity motor nerve stimulation potentiates the tonic adenosine $A_{2A}R$-mediated facilitation of ACh release, due to accumulation of ADO in the synaptic cleft, which may overcome muscular tetanic fade, whereas activation of the inhibitory A_1R becomes less effective (Correia-de-Sá *et al.* 1996). During 50 Hz-trains, ATP is able to reach high levels, enough to inhibit CD73. Interburst intervals, allows the recovery from CD73 enzymatic inhibition, because there is a delayed burst-like formation of ADO, leading to high synaptic concentrations of ADO, similar to those required to promote the activation of $A_{2A}R$ (Correia-de-Sá *et al.* 1996).

$A_{2A}R$ act via subtle modifications of the presynaptic inter-receptor dynamics (Sebastião & Ribeiro 2000) involving the generation of intracellular second messengers, such as cAMP (Correia-de-Sá & Ribeiro 1994) and Ca^{2+} (Correia-de-Sá *et al.* 2000). It worth noting that fine-tuning control of facilitatory nAChRs containing α3β2 subunits (Faria *et al.* 2003) and muscarinic M_1 and M_2 (Oliveira *et al.* 2002) receptors, is mediated by endogenous ADO. In parallel, there is a co-ordinate shift in Ca^{2+} cell dynamics operating ACh exocytosis, from the prevalent P/Q-type to the "facilitatory" L-type channel, in a way completely reversed by blocking $A_{2A}R$ activation (Oliveira *et al.* 2004). These mechanisms represent a novel form of synaptic plasticity mediated by ADO and may function to overcome neuromuscular tetanic depression during neuronal firing.

Neurotransmission failure in MG is particularly evident during intense motor nerve activity, a situation where ADO, acting via $A_{2A}R$, has a key role by promoting increases in the safety margin of NMT (Correia-de-Sá & Ribeiro 1996).

Recently, our group demonstrated that $A_{2A}R$ fine-tuning control of NMT is impaired in animals models of MG (Noronha-Matos *et al.* 2011; Oliveira *et al.* 2015b). This seems to be mainly due to a decrease in endogenous ADO levels, leading to a reduction on tonic $A_{2A}R$ activity, which can be functionally recovered by application of the ADO precursor, AMP (Noronha-Matos *et al.* 2011; Oliveira *et al.* 2015b). These findings led us to propose that changes in $A_{2A}R$ signaling dynamics might be associated to the pathogenesis of MG, therefore exploring new mechanisms to recover endogenous ADO amounts could be of clinical interest.

3. Aim

Nowadays, therapeutic strategies to control MG are mainly devoted to counteract immune response hyperactivity. To this end, corticosteroids are considered first line medications. Recently, our group demonstrated that methylprednisolone (MP 300 µM) increases ATP release

above baseline from resting motor endplates, which anticipates ADO accumulation at the synaptic cleft, thus contributing to amplify NMT via the activation of presynaptic facilitatory $A_{2A}R$ in healthy animals (Oliveira *et al*. 2015a). Sustained transmitter release due to facilitatory $A_{2A}R$ activation has been previously shown as an important mechanism to overcome tetanic depression in healthy individuals (Oliveira *et al.* 2004). Considering the data obtained in MG animal models, demonstrating that endogenous ADO generated in myasthenic motor endplates, during repetitive nerve stimulation is insufficient to sustain transmitter release demand through tonic activation of presynaptic facilitatory $A_{2A}R$ (Noronha-Matos *et al.* 2011; Oliveira *et al.* 2015a), we hypothesized that corticosteroid benefits (avoidance of neuromuscular failure) in patients with MG may result from a mechanism that rehabilitates endogenous ADO accumulation at the neuromuscular synapse to levels high enough to activate presynaptic facilitatory $A_{2A}R$, leading to increases in ACh release and muscular strength. The role of MP in the control of NMT and ATP release was evaluated by conventional neurochemical techniques (see e.g. Correia-de-Sá *et al.* 1991) and the ATP outflow was quantified by the luciferin – luciferase ATP bioluminescence (Oliveira *et al*. 2015a).

4. Materials and methods

4.1. Induction and clinical assessment of Experimental Autoimmune Myasthenia gravis (EAMG) rat models

Females rats (*Wistar Han*) (Charles River, Barcelona, Spain), were kept at a constant temperature (21ºC) and a regular light (07.00–19.00 h) – dark (19.00 – 07.00 h) cycle, with food and water *ad libitum*. These animals were handled as stated in the Portuguese Decree - Law nº 113/2013 of 7th August, concerning the protection of animals, used for experimental procedures and for other scientific purposes (Hau & Schapiro 2011). Their handling and experiments, were also in accordance with the guidelines prepared by the Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). These animals were randomly divided into three groups: Naïve, Control and Myasthenic (EAMG) groups. These animals were induced, and maintained in the vivarium according to the rules of Biotério ICBAS – UP.

Under general anesthesia with the anesthetic medetomidine (0.5 mg/kg intraperitoneal (ip) /ketamine (75 - 90 mg/kg ip) combination (Rosenthal *et al.* 2008; Turner 2011; Papich 2011; Carpenter & Marion 2013), the rats in the EAMG group, were immunized by subcutaneous injection at six sites (two plantar regions, shoulders and bilaterally in the lower back) with 50 microgram (µg) of R97-116 peptide (DGDFAIVKFTKVLLDYTGHI, JPT Peptide Technologies GmbH) – a synthetic peptide, corresponding to the region 97-116 of the rat nicotinic AChR α

subunit – emulsified in 200 microliters (µL) Complete Freund's Adjuvant (CFA) (Sigma, St. Louis, MO, USA). Injections were performed on day 0 and were boosted on day 30 with the same peptide in Incomplete Freund's adjuvant (Incomplete Freund's Adjuvant (IFA) - lacks of *Mycobacterium tuberculosis* - 200 µL) (Baggi *et al.* 2004; Oliveira *et al.* 2015b). The Control group was immunized with CFA and IFA emulsions, respectively, containing phosphate-buffered saline (PBS) instead of the nAChR R97-116 peptide at the respective time points. Animals in the Naïve group were left untreated.

Each animal was weighed and evaluated for disease manifestation twice weekly until euthanized by decapitation on day 42 post immunization (pi) (Baggi *et al.* 2004; Oliveira *et al.* 2015b). On the evaluation days, all the animals included in this study, were submitted to the same experimental procedure, and were examined equally. Evaluation of disease manifestations in immunized rats was performed by testing muscular weakness. Clinical scoring was based on the presence of tremor and hunched posture and muscle strength by grip strength test (BIOSEB, France), and fatigability was assessed after exercise (repetitive paw grips on the cage grid). Disease severity was graded using the follow clinical scoring: grade 0, normal strength and no muscle weakness; grade 1, normal at rest, but weak after exercise (chin on the floor; inability to raise head; hunched back); grade 2, clinical signs at rest; grade 3, severe clinical signs at rest, moribund, dehydrated and quadriplegic; and grade 4, dead (Baggi *et al*. 2004). The animals were also evaluated for loss of body weight, and for animal welfare critical points (Meredith & Redrobe 2002; Quesenberry & Carpenter 2012).

The expression "Humane endpoints" becomes extremely important, and since "there are ethical, scientific and legal reasons for ensuring that adverse effects are minimized", the "choice of appropriate humane endpoints provides significant opportunities for refinement, and should be developed in tandem with the requirements for a valid scientific outcome. Early endpoints reduce non-specific systemic effects and so may increase the precision of the results obtained." (Workman *et al.* 2010) A new table of "Humane endpoints" was created based on the most common signals observed in the EAMG animals induced in the new vivarium. Was also adjusted to the alterations in the respiratory system observed, post grip strength test, and the induced behavior after manipulation of the animals (Attachment 2). A proper knowledge of the biology of these animals (*Rattus norvegicus*) is mandatory, to recognize signals of stress, pain, or any other unexpected behavior, and therefore has been taken into account in the development of the new table (Meredith & Redrobe 2002; Quesenberry & Carpenter 2012; Hau & Schapiro 2011). These "Humane endpoints" are in accordance with Portuguese Decree - Law nº 113/2013 of 7th August (Hau & Schapiro 2011), the guidelines prepared by the National

Research Council, USA, the guidelines of the Canadian Council On Animal Care (Olfert *et al.*1998), and the European Communities Council Directive (86/609/EEC).

The animals were monitored for body weight, and for animal welfare critical points twice a week, except for the first 3 to 5 days, after the first and second boost which were monitored on a daily basis. A decreased or maintenance of body weight in the day 1 after the induction and also in the next day of the second boost (day 31), was observed in both the Control and EAMG groups. This was only due to decreased food intake during this period. Nevertheless, in Figure 3 which illustrates the weight variation over time, shows no significant differences, in the growth curves from the three groups of animals (Naïve *n*=3; Control *n*=27; EAMG *n*=29), which indicate that the muscle weakness, commonly observed in EAMG, did not interfere with the normal food intake. Between days 10 – 20 pi, all groups experienced weight loss, which we do not have a reasonable explanation for.

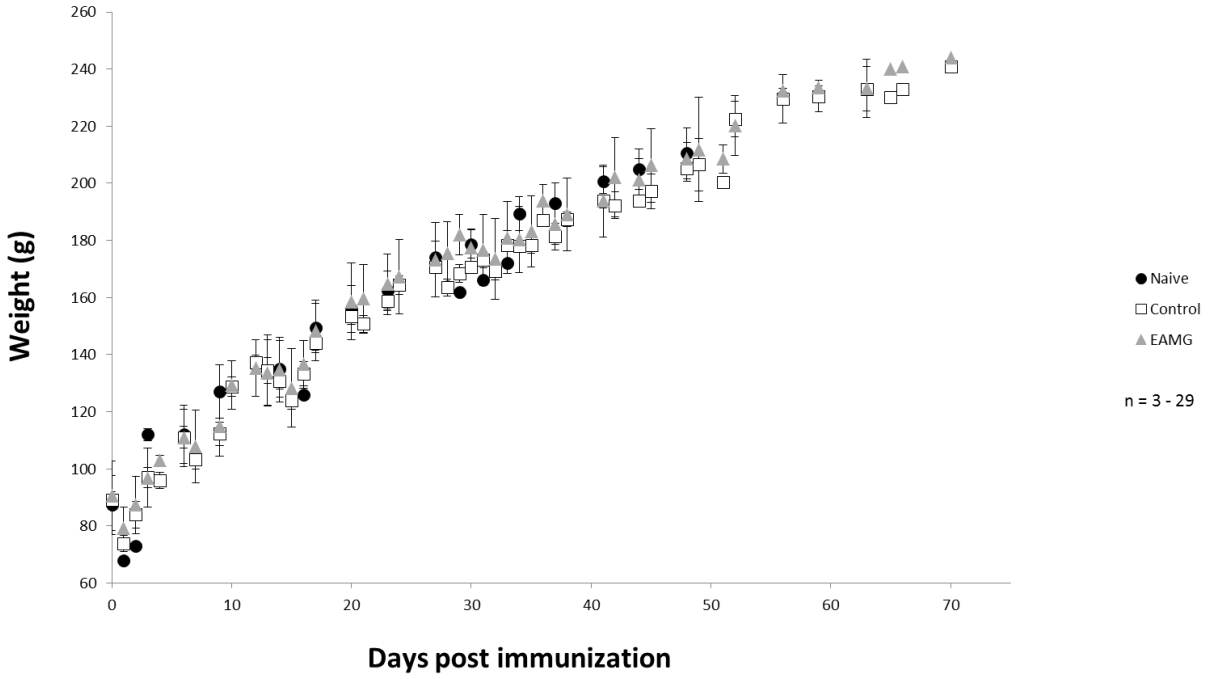

Figure 3 – The weight variation over time (days pi) shows no significant differences, in the growth curves from the three groups of animals (Naïve ● *n*=3; Control □ *n*=27; EAMG▲ *n*=29). All the results are presented as mean +/- Mean standard error (SEM) .The vertical bars represent ± SEM.

The onset and progression of the disease is expressed in terms of mean +/- SEM of the welfare score, and is illustrated in Figure 4 according with the new table of “Humane endpoints”, where the welfare of the animals was graded as follows: 0 – 4: Normal; 5 – 13: kept close attention and increase the frequency of motorization; 14 or more: pain, required veterinarian observation

and supportive care. Rats immunized with R97 – 116 peptide, presented a higher score (Figure 4), with a medium value of 3.9 between days 0 – 30 pi, and 4.1 after day 30 pi (Table 1), and it is also observed that the Control group, with an average of 2.8 between days 0 – 30 pi, and 3.2 after day 30 pi (Table 1), has levels of score, significant higher than the Naïve group (which have an average of 0,58 between days 0 – 30 pi, and 1.1 after day 30 pi) (Naïve *n*=3; Control *n*=27; EAMG *n*=29) (Table 1).

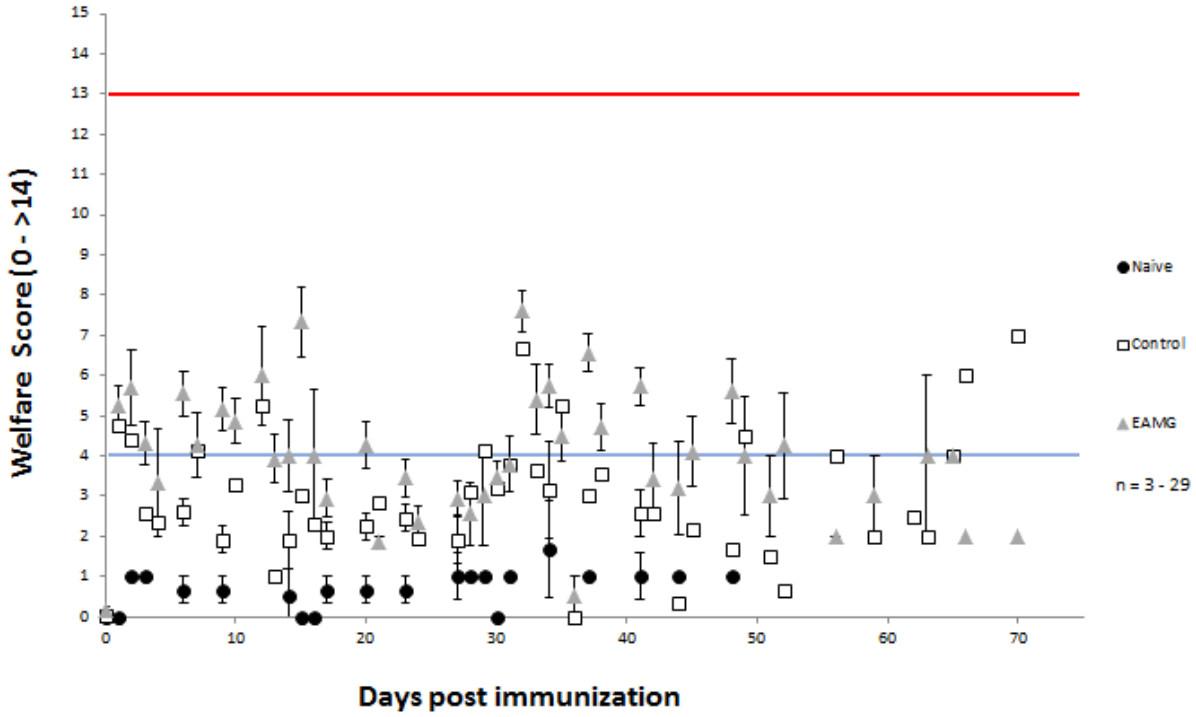

Figure 4 – Clinical score of the three groups over time (days pi) - shows differences, between the groups of animals (Naïve ● *n*=3; Control □ *n*=27; EAMG▲ *n*=29). All the results are presented as mean +/- SEM (the vertical bars represent ± SEM).

In Table 1 are presented the media values of the welfare score of these animals.

	Day 0 – 30 pi	After day 30 pi
Naïve	0,58	1,1
CFA	2,8	3,2
EAMG	3,9	4,1

Table 1 – Welfare average values of the clinical score obtained in the period between day 0 – 30 pi and after day 30 pi. All animals were, in general, in the category - Normal (Naïve *n*=3; Control *n*=27; EAMG *n*=29).

The slight decreased or maintenance of the body weight previously referred, in both Control and EAMG groups, is coincident to the increase in the welfare score of these animals. These changes are a result of the sc inoculations inherent to the inducing protocol of EAMG, and the development and progression of the disease, that causes some discomfort. Despite that, the welfare average of the three groups is well below the alarming categories of the new table of "Humane endpoints" and the majority of the animals along the protocol demonstrated a normal behavior and welfare.

4.2 Preparation and experimental conditions

All animals were euthanized by decapitation, using a guillotine, a fast method, which has the advantage of allowing a good exsanguination; this may be required to enable the collection of blood samples for subsequent procedures. Then, the animals were submitted to surgical isolation of the phrenic nerve hemidiaphragm as described by Correia-de-Sá and Collaborators (1991) (Figure 5).

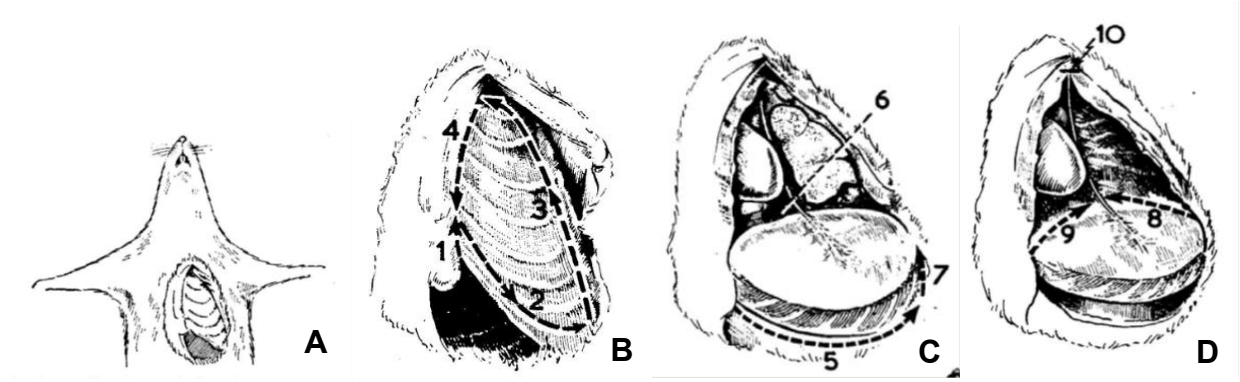

Figure 5 – Surgical isolation of the phrenic nerve hemidiaphragm as described by Correia-de-Sá and Collaborators (1991), adapted from Oliveira 2013: **A -** Incision in the thoracic region; **B -** Removal of the ribs; **C -** Removal of parietal pleura; **D -** Isolation of phrenic nerve hemidiaphragm and dissection of the innerved diaphragm.

The experiments were performed using either left or right phrenic nerve-hemidiaphragm preparations (4 - 6 millimeters (mm) width). Each muscle was superfused (5 $mL.min^{-1}$, 37°C, pH 7.4) with gassed (95% O_2; 5% CO_2) Tyrode´s solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 11.2, at 37ºC (Correia-de-Sá *et al.* 1991).

4.3. [^{3}H]-ACh release experiment from phrenic nerve hemidiaphragm preparations

The procedures used for labeling the preparations and measuring evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release, have been previously described (Correia-de-Sá *et al*. 1991). Briefly, phrenic nerve-hemidiaphragm preparations were mounted in 3-mL capacity Perspex chambers heated to 37ºC. After a 30 min equilibration period, the perfusion was stopped and the nerve terminals

were labeled for 40 min with 1µM [^{3}H]-choline (specific activity 2.5 µCi/nmol) under electrical stimulation, at a frequency of 1 Hz (0.04ms duration, 8mA). The phrenic nerve was stimulated with a glass–platinum suction electrode, placed near the first division branch of the nerve trunk, to avoid direct contact with muscle fibres (Figure 6).

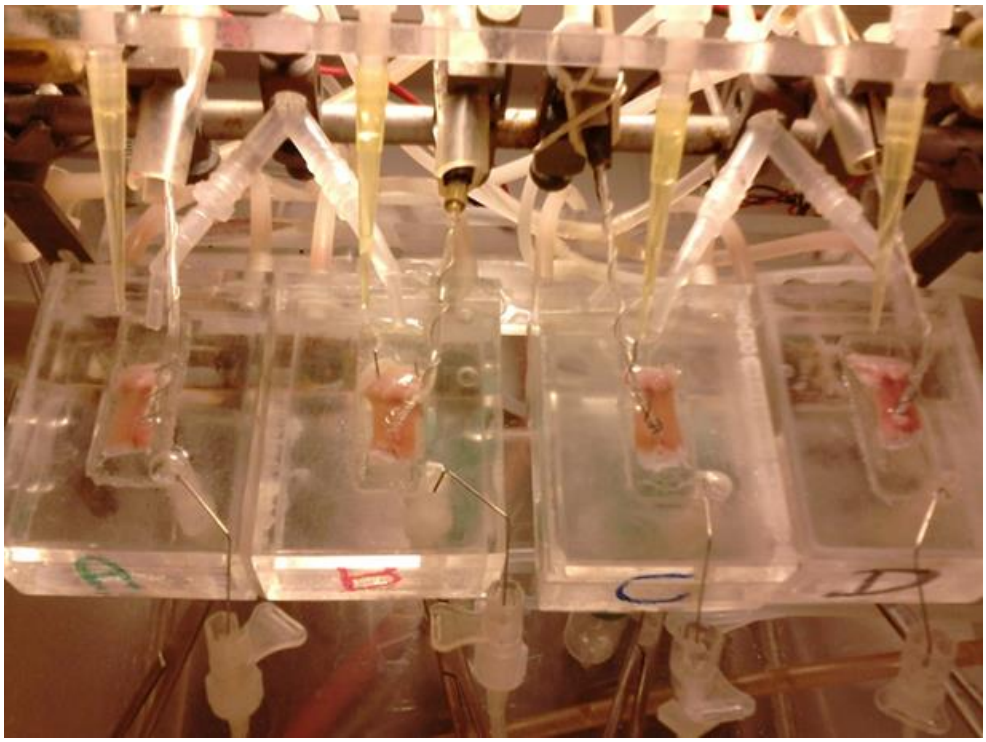

Figure 6 – Photography of the isolated phrenic nerve-hemidiaphragm preparations mounted horizontally in thermostatized organ bathes used to quantify the release of [^{3}H]-ACh release experiment - isolated phrenic nerve hemidiaphragms preparations. Each phrenic nerve was inserted inside a suction electrode manufactured in the laboratory.

After the labeling period, the preparations were again superfused (37.5 mL/min) and the nerve stimulation ceased. From this point onwards, hemicholinium-3 (10 µM) was present to prevent the uptake of [^{3}H]-choline and the synthesis of unlabeled ACh. After a 60 min washout period 1.5 mL, bath samples were automatically collected every 3 min using a fraction collector (Gilson, FC 203B, France) coupled with a peristaltic pump (Gilson, Minipuls3, France) programmed device by emptying and refilling the organ bath with the solution in use. The release of [^{3}H]-ACh was evoked by two periods of electrical stimulation of the phrenic nerve, starting at min 12 (S_1) and min 39 (S_2), after the end of washout (zero time). Supramaximal intensity rectangular pulses (0.04 ms duration, 8 mA) were delivered at 50 Hz frequency. A series of 5 bursts of 150 pulses were applied, with a 20 s interburst interval - tetanic stimulation (Correia-de-Sá *et al.* 1996). Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Medium incubation aliquots (0.4 mL) were added to 3.5 mL of Packard Insta Gel II (USA) scintillation cocktail so that tritium content samples could be

measured by liquid scintillation spectrometry (counting efficiency of 40±2%). Radioactivity is expressed as DPM (disintegrations per minute). The evoked release of [^{3}H]-ACh was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (Correia-de-Sá *et al.* 1996). The change in the ratio between the evoked [^{3}H]-ACh released during the two stimulation periods (S_2/S_1), relative to the observed in control situations (in the absence of test drugs) was taken as a measure of drugs effects.

4.4. Release of basal ATP from phrenic nerve hemidiaphragm preparations

For ATP release experiments, the innervated hemidiaphragm preparations were mounted as described previously for radiochemical experiments. After a 30 min equilibration period, the perfusion was stopped and aliquots of 1.5 mL bath samples collected automatically every 3 min. During the first 6 min, the preparations were incubated with Tyrode´s solution; then MP 300 µM was added to the Tyrode's solution for the next 24 min. Every 3 min samples were collected and the chambers refilled with fresh solution. Two hundred microliter aliquots were introduced into pre-cooled microtubes, which were frozen in liquid nitrogen until analysis The ATP content of the samples was evaluated by the luciferin – luciferase ATP bioluminescence assay kit HS II (Roche Applied Science, Indianapolis, Indiana). Luminescence was determined using a multi detection microplate reader (Synergy HT, BioTek Instruments) (Oliveira *et al*. 2015a).

4.5. Drugs and Solutions

Methylprednisolone sodium succinate (Solumedrol ^{TM}, Pfizer); choline chloride; hemicholinium-3; CFA and IFA were obtained from Sigma, St. Louis, MO, USA; 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]t (ZM 241385) (Tocris Bioscience, Bristol, UK), made up in dimethylsulphoxide; [methyl-^{3}H]choline chloride (ethanol solution, 80.6 Ci mmol^{-1}) and the scintillation cocktail (Insta – gel Plus) were obtained from Perkin Elmer (Boston, USA); R97-116 peptide (DGDFAIVKFTKVLLDYTGHI) was obtained from JPT Peptide Technologies GmbH. All stock solutions were stored as frozen aliquots at - 20ºC. Dilutions of these stock solutions were made daily.

5. Results and Discussion

5.1. Methylprednisolone-induced facilitation of transmitter exocytosis depends on tonic activation of A_{2A}R on motor nerve terminals of EAMG rats

Methylprednisolone (MP) is a glucocorticoid used in some neurological cases, both in human and veterinary medicine (Dickinson 2012).

Despite corticosteroids have been used for decades in the treatment of autoimmune myasthenic syndromes, the mechanism underlying the improvement of NMT is still a matter of debate.

Recently, our group demonstrated that in healthy (Naïve) motor endplates, tetanic depression could be overcome by MP-induced facilitation of transmitter release (Oliveira *et al.* 2015a). Such a mechanism would be clinically relevant to explain corticosteroid benefits (avoidance of neuromuscular tetanic failure) in patients with *Myasthenia Gravis*. In this context, we decided to evaluate the effects of MP (300 µM) on evoked [^{3}H]-ACh release during 50 Hz stimulation bursts of phrenic nerve hemidiaphragm preparation obtained from EAMG animals.

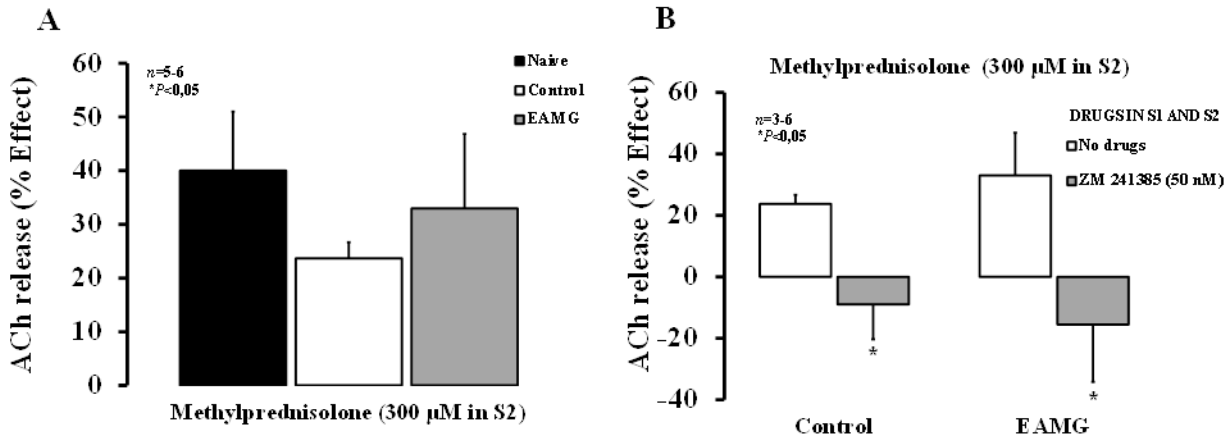

Figure 7 – Facilitation of nerve-evoked [^{3}H]-ACh release by methylprednisolone (MP) (300 µM), is mediated by tonic activation of $A_{2A}R$ on motor nerve terminals. Transmitter release was elicited by stimulation with 50 Hz Bursts of the phrenic nerve trunk (5 trains of 150 pulses applied with a 20 seconds interburst interval). Experiments were performed 6 weeks after immunization with the peptide R97-116 corresponding to the α-subunit of nAChR in CFA (EAMG), as compared to age-matched Naïve and Control littermates. **A** - MP was applied 15 min before the second period of stimulation (S_2). The ordinates represent percentage of increase in the S_2/S_1 ratio, caused by MP (300 µM) application. **B** - MP was applied 15 min before the second period of stimulation (S_2) either in the absence or presence of the selective $A_{2A}R$ antagonist, ZM 241385 (50 nM), which was present in S_1 and S_2. The ordinates represent the percentage of increase in the S_2/S_1 ratio, caused by MP (300 µM) application. The vertical bars represent mean ± SEM $p^* < 0.05$ (Mann-Whitney test) when comparing the effect of MP in the presence and absence of ZM 241385.

Incubation of hemidiaphragm preparations from Control and EAMG rats with MP (300 µM) 15 min before S_2 increased significantly ($p<0.05$) [^{3}H]-ACh release triggered by phrenic nerve stimulation with 50 Hz bursts (150 pulses applied 5 times with a 20 s interburst interval) (Figure 7-A). No significant changes ($p>0.05$) were detected when comparing the facilitatory effect of MP (300 µM) in EAMG rats (33±1%, n=5) with Controls (24±3%, n=6) and Naïve (40±11%, n=5) animals. Since the methylprednisolone-induced tetanic enhancement of NMT in Naïve animals depends on tonic activation of presynaptic facilitatory $A_{2A}R$ (Oliveira *et al.* 2015a) we designed experiments to test if these receptors are also involved in corticosteroid induced amplification of NMT in myasthenic animals. Experiments were designed to evaluate the effect of MP (300 µM)

on evoked [^{3}H]-ACh release induced by 50 Hz bursts in the presence of the selective $A_{2A}R$ antagonist, ZM241385 (50 nM). As observed in Naïve rats, amplification of transmitter release by the MP (300 µM) depend on tonic $A_{2A}R$ activation because pre-treatment of hemidiaphragm preparations with ZM241385 (50 nM) prevented the facilitatory effect of MP (300 µM) in both control (-9±11%, *n*=3) and EAMG (-16±19%, *n*=3) animals (Figure 7-B).

In observational studies, remission or marked improvement is seen in 70–80% of patients with MG, treated with oral corticosteroids indicating that therapeutic approaches using corticosteroids are highly effective (Sathasivam 2008). Until now the clinical benefits of corticosteroids in MG are mainly attributed to their immunosuppressive effects. Steroids are thought to inhibit the activation of T cells by interfering with the activation process in the cell nucleus (Allison 2000). In addition, steroids impair the function of cells of the monocyte–macrophage lineage by inhibiting antigen processing and decreasing the number of circulating T cells (Taylor *et al*. 2005). In this work, we show for the first time that in addition to their immunosuppressive action corticosteroids can improve the clinical features of MG. The corticosteroid, MP, can rehabilitate NMT failure in EAMG animals by amplifying neurotransmitter release.

Typically, MG patients present with a history of weakness and fatigability of muscles on sustained or repeated activity which corresponds to nerve stimulation increases from 5 to 50 Hz (Conti-Fine *et al*. 2006; Hirsch 2007). In this context our results gain pathophysiological interest since the beneficial effects of corticosteroids operates only at nerve stimulation frequencies of 50 Hz-Bursts (Oliveira *et al* 2015a). At high frequency (50 Hz) stimulation, a coordinated shift from a prevailing A_1-inibitory towards A_{2A}-facilitatory tonus on NMT modulation occurs at healthy motor nerve endplates (Correia-de-Sá *et al.* 1996). This constitutes an important mechanism to sustain transmitter release and to overcome tetanic depression in healthy conditions (Oliveira *et al*. 2004). However, in myasthenic motor nerve endplates the sustained transmitter release mediated by tonic $A_{2A}R$ activation is impaired (Oliveira *et al.* 2015b). Interestingly, in this work we demonstrated that the corticosteroid also exerts its beneficial effect on myasthenic animals by recruiting the presynaptic facilitatory $A_{2A}R$.

Moreover, we showed that in healthy animals amplification of NMT by MP involves a predominant activation of presynaptic facilitatory $A_{2A}R$, leading to synaptic vesicles redistribution favoring transmitter exocytosis during high-frequency neuronal firing. Whether this mechanism underlying endo/exocytosis modifications operates in EAMG motor nerve terminals requires further investigations.

Due to their immunosuppressive and neuromodulatory actions pharmacological targeting of $A_{2A}R$ activity constitutes an appealing therapeutic strategy for MG patients (Oliveira *et al.* 2015a). We have recently demonstrated that insufficient amounts of ADO to promote immune cells immunosuppression and NMT via $A_{2A}R$ activation occur in EAMG animals (Oliveira *et al.* 2015b). In Naïve rats tonic activation of facilitatory $A_{2A}R$ by endogenous ADO generated from ATP released, under resting conditions, is vital for methylprednisolone-induced facilitation of transmitter release during high-frequency bursts (Oliveira *et al.* 2015a). In fact, increments of extracellular ATP derived ADO formation favoring $A_{2A}R$ activation may be crucial to rehabilitate cell communication deficits in myasthenics, since immune suppression and NMT deficits in EAMG animals may be rehabilitated by $A_{2A}R$ activation by exogenous AMP application serving as an ADO precursor (Oliveira *et al.* 2015b).

In this context we decided to investigate the time course of MP (300 µM) induced ATP release in non-stimulated phrenic nerve hemidiaphragm preparations collected from all animal groups, using the luciferin-luciferase bioluminescence assay.

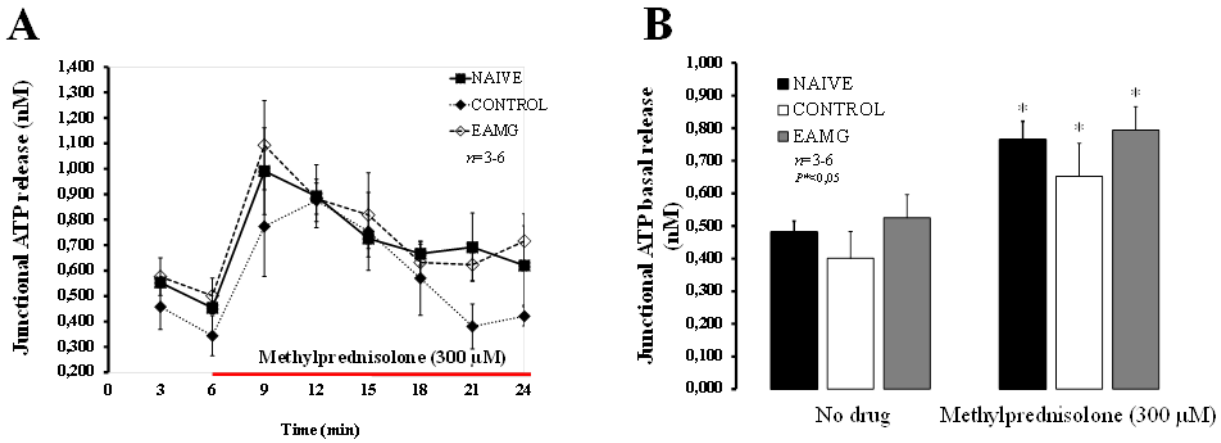

Figure 8 - Increase in the resting ATP outflow at the rat neuromuscular junction, induced by methylprednisolone (300 µM). **A** – Time-course of basal ATP outflow from innervated hemidiaphragm preparations in the presence or absence of MP (300 µM) collected from rats immunized with the peptide R97-116 corresponding to the α-subunit of nAChR in CFA (EAMG), and to age-matched Naïve and Control littermates. Abscissa indicates the times at which samples were collected. **B** – Average mean value of basal ATP outflow in the presence and absence of MP (300 µM) for all animal groups. $p^* < 0.05$ (Mann-Whitney test) when comparing the basal ATP release induced by MP (300 µM) application with basal ATP release in the control conditions.

In all animal groups, application of MP (300 µM) rapidly increase basal ATP outflow from phrenic nerve-hemidiaphragm preparations (Figure 8-A). The maximum peak in basal ATP release was observed 3 min after MP (300 µM) application in Naïve (991±170 pM) and EAMG animals (1093±175 pM) (Figure 8-A). Interestingly, the increase in ATP outflow was kept at high levels for the time of MP (300 µM) application (18 min) in all groups of animals (Figure 8-A). The

average basal ATP outflow during MP (300 µM, 18 min) application was always higher than baseline in Naïve (765±57 vs 481±33 pM *n*=3), Control (653±101 vs 401±82 pM *n*=6) and EAMG (794±71 vs 526±71 pM *n*=5) animals (Figure 8-B).

Data show here that MP can rehabilitate NMT failure in EAMG animals by increasing the outflow of ATP from resting motor endplates, which upon its extracellular hydrolysis into ADO may increase the $A_{2A}R$ tonus, leading to facilitation of evoked transmitter release during high-frequency nerve firing.

ATP can be released to the extracellular compartment by non-lytic mechanisms including: (1) exocytosis of ATP-containing vesicles; 2) through nucleotide-permeable channels (connexin and pannexin hemichannels, maxi-anion channels, volume-regulated anion channels or P2X7 receptor channels); (3) via transport vesicles that deliver proteins to the cell membrane; (4) via lysosomes. The contribution of these mechanisms on MP induced ATP outflow deserves further investigations. Taking into consideration that long-term use of corticosteroids is associated with many adverse events, including Cushingoid symptoms, infections, hypertension, diabetes, osteoporosis, psychiatric disorders, insomnia, and elevations in white blood cell count, the need of achieving new pharmacological target with the same efficiency and with less side effects emerges. As stated previously pharmacological targeting of $A_{2A}R$ activity in MG constitutes an appealing target. So, understanding the mechanisms associated to MP induced ATP release and $A_{2A}R$ activity may give important insights to unravel new pharmacological strategies for MG treatment.

6. Conclusion

Recent research has increases our understanding of NMT, which allowed the development of better diagnostic techniques to identify MG patients sooner, providing the establishment of supportive therapy in the earlier state of the disease. The conventional method of treatment with AChE inhibitors is still a viable treatment, but in severe cases early in the course of MG, may be required immunosuppressive drugs, which sometimes are insufficiently to control the symptoms during inflammatory crisis (Khorzad *et al.* 2011). It is clear that randomized controlled trials are necessary to determinate the most effective therapy.

Neurotransmission failure in MG is particularly evident during intense motor nerve activity, a situation where ADO, acting via $A_{2A}R$, has a key role by promoting increases in the safety margin of NMT (Correia-de-Sá & Ribeiro 1996). Our group provided evidence showing that tonic activation of presynaptic facilitatory $A_{2A}R$ may contribute to overcome tetanic depression during high-frequency neuronal firing in healthy rats (Oliveira *et al.* 2004), we strongly believe that presynaptic manipulation of $A_{2A}R$ activity might prove therapeutically useful for myasthenic

syndromes. In fact, a dysfunction on $A_{2A}R$ activity seems to contribute to the pathophysiology of myasthenic syndromes, since during nerve stimulation endogenous ADO generated in myasthenic motor endplates is insufficient to sustain transmitter release demand through tonic activation of presynaptic facilitatory $A_{2A}R$ (Noronha-Matos *et al.* 2011; Oliveira *et al.* 2015a). In this work we demonstrated that corticosteroid commoly used in the treatment of MG increases ATP release above baseline from resting myasthenic motor endplates, which anticipates ADO accumulation at the synaptic cleft, thus contributing to amplify NMT via the activation of presynaptic facilitatory $A_{2A}R$, leading to transmitter exocytosis during high-frequency neuronal firing. These findings contribute to elucidate the non-immunological beneficial effects of corticosteroids in NMT deficits, and confirm that ADO path may be a key target for therapeutic intervention in MG (Oliveira *et al*. 2015b).

The mechanism underlying non-genomic glucocorticoid-induced ATP release from innervated skeletal muscles deserves further elucidation.

Further insights on some of these issues would allow a better understanding of the biochemical and molecular mechanisms involved in the pathophysiology of ADO signaling pathways in myasthenic disorders and, perhaps, would unravel new avenues towards novel therapeutic approaches for myasthenic syndromes.

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8. Attachments

Attachment 1 - Similarities and differences between MG and EAMG. Adapted from Baggi *et al.* 2012.

	Similarities	Differences
Immunopathological features	Presence of anti-AChR Abs in the serum; Deposits of IgGs and C3 complement component at the NMJ; Loss of muscle nAChRs; MHC class II-restricted presentation of AChR epitopes; Involvement of T helper cells in B - cell antibody production.	Disease does not arise spontaneously in animals, needs for induction factors; Involvement of the thymus (present in some cases of MG, absent in EAMG). Thymic alterations are absent in EAMG, and in MG patients, hypertrophy and thymomas are often present; Phagocytic cells detected in the acute phase of rat EAMG, are absent at the NMJ of human MG patients.
Clinical manifestations	Muscle weakness, most prominent in the upper body; Decreased response in the repetitive nerve stimulation test; Reduction in the miniature end-plate potential amplitude; Temporary improvement in muscle strength after anti-AChE treatment (Tensilon test).	Absence of ocular signs; Absence of relapse and remission periods.

MG patients and EAMG share several features, in particular muscle weakness, fatigability and decremented response after repetitive nerve stimulation. When treated with anti-cholinesterase drugs occurs a temporary improvement of strength, and appear similarities in many immunopathological features, such as presence of anti-AChR Abs in serum, deposition of IgGs at the NMJ, MHC II presentation of AchR epitopes and involvement of T helper cells in B cell Abs production (Baggi *et al.* 2012). Also, in contrast with MG in humans, the disease is not spontaneously in animals (Baggi *et al.* 2004).

Attachment 2 - Table to evaluate the "Humane Endpoints" of the "Projecto nº 174 – Miastenias" (Oliveira & Mota 2014)

Data de Indução: Boost 1 — Boost 2 — **Tabela para avaliação de "Humane Endpoints" PROJETO: 174 - Miastenias**

Data de nascimento: — Sexo: — Ninhada: — Dia teste

Tratamento/ ID animal: — Data — Peso(g)

Parâmetro	Características do animal	Score																			
Aparência Geral	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Alte Anatomia/ Desidratação/ Feridas/ Hipotermia/ Hipertermia/Dor/Alopécia	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2 dos sinais indicados anteriormente	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	3 dos sinais indicados anteriormente	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	4 dos sinais indicados anteriormente	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Pêlo, pele e olhos	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Granuloma, Falta geral de manutenção do pêlo, Cromodacriorreia ligeira	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2 dos sinais indicados anteriormente	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	anteriores/ Cromadocriorreia Grave, Piloereçao	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	anterior + olhos semi-cerrados + caquéxia	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Região plantar	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Alteração da Anatomia/ Edema/ Eritema/ Calor/ Dor/ Descamação/ Ferida	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2 dos sinais indicados anteriormente	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	3 dos sinais indicados anteriormente	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	4 dos sinais indicados anteriormente	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Comportamento espontâneo	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Alterações menores (redução no Grooming, diminuição da Curiosidade)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Isolamento e mobilidade reduzida, mas alerta	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Inquieto, Vocalização, Tremores, Agressividade ou muito Parado	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	Não alerta (imóvel), não responsivo)	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Nutrição/perda de condição corporal	Normal (peso normal ~ controlo)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderada (redução semanal de 10% face ao controlo)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Severa (Redução semanal de 15% face ao controlo)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Crítica (Redução semanal de 20% face ao controlo)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	Extrema (redução de 20% face ao peso do próprio animal da última pesagem)	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Sinais clínicos : antes grip test	Ritmo e padrão respiratório normais	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Alteração modesta, apenas ritmo respiratório aumentado	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Ritmo respiratório acelerado com respiração abdominal	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Ritmo respiratório reduzido com respiração abdominal	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	Respiração abdominal lenta e pronunciada, com cianose e hipotermia	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Sinais clínicos : após grip test	Ritmo e padrão respiratório normais	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Alteração modesta, apenas ritmo respiratório aumentado	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Ritmo respiratório acelerado com respiração abdominal	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Ritmo respiratório reduzido com respiração abdominal	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	Respiração abdominal lenta e pronunciada, com cianose e hipotermia	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Comportamento induzido	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Resposta Reduzida ou exagerada (Fuga,Vocalização,Tremores, Agressividade)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Resposta muito reduzida e lenta	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Resposta Inexistente com hipotermia (estado pré-comatoso)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Score Extra	Adicionar **+1** por cada 3 e **+2** por cada 4																				
	TOTAL																				

0 - 4 : Normal

5 - 13 : Vigiar com atenção, aumentar frequência de monitorizações

14 ou mais : Sofrimento, avaliação pelo Veterinário e medidas suporte

Observações